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Sinorhizobium meliloti ExoR Is the Target of Periplasmic Proteolysis

Hai-Yang Lu, Li Luo, Meng-Hua Yang, and Hai-Ping Cheng

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the Gram-negative soil bacterium Sinorhizobium meliloti establishes a nitrogen-fixing symbiosis with its plant host, alfalfa (Medicago sativa), through a set of complex and reciprocal signal exchanges in the absence of fixed nitrogen sources (11, 17, 27, 32, 48). The formation of an infection thread inside alfalfa root hairs is an essential step in the early stage of this symbiosis that requires the presence of S. meliloti exopolysaccharides, succinoglycan (SG), exopolysaccharide II (EPSII), or capsular polysaccharide (KPS). S. meliloti SG has been shown to be much more effective than the other two S. meliloti polysaccharides, EPSII and KPS, at eliciting the formation of infection threads (3, 7, 23, 29, 39). The structure and biosynthetic pathway of succinoglycan have been well documented, although its precise role in eliciting the formation of infection threads remains unknown (20–22, 30, 43, 51).

Succinoglycan production is inversely coregulated with flagellum production by a single signal transduction pathway consisting of the S. meliloti ExoS protein and the ExoS/ChvI two-component regulatory system (55) and the EmmABC system (37). While the transcription of succinoglycan biosynthesis genes is upregulated by the mutations exoR95::Tn5 and exoS96::Tn5, transcription of the flagellum biosynthesis genes is downregulated (55). This coordinated regulation is consistent with the switch from free-living to invasion-ready cells that is required at this stage of symbiosis. It also suggests that the ExoR protein and the ExoS/ChvI two-component system play crucial roles in controlling the overall changes needed for S. meliloti cells to switch from free living to symbiosis inside the root nodules.

The S. meliloti exoR gene was initially identified through isolation of the exoR95::Tn5 mutation, which was later identified and sequenced (10, 41). The exoR gene encodes a 268-amino acid ExoR protein with a conserved signal peptide for exporting the protein to the bacterial periplasm, as confirmed in recent findings (53). In addition to regulating succinoglycan and flagellum production, ExoR has been shown to be involved in regulating biofilm production and lipopolysaccharide modifications (16, 28). The ExoR protein has been found to regulate the expression of a large number of gene functions in very different metabolic pathways, suggesting that ExoR plays other important roles (53). ExoR homologs have been found and characterized in Rhizobium leguminosarum and Agrobacterium tumefaciens, where they also function in regulating polysaccharide, flagellum, and biofilm production (42, 47). Many additional ExoR homologs have been discovered in recent genome-sequencing efforts, but little is known about their functions.

The S. meliloti ExoS and ChvI proteins form a typical bacterial two-component signal transduction system (8, 38). The S. meliloti ExoS protein consists of a large periplasmic domain and a cytoplasmic kinase domain, and it has been shown to phosphorylate S. meliloti ChvI directly (8). Recent analysis of exoS and chvI deletion mutants has shown that the ExoS/ChvI system is essential for symbiosis and that these two proteins regulate the expression of a variety of genes involved in carbon metabolism and many other functions (2, 50). These findings are consistent with the results of a transcriptome analysis of the exoR96 mutant (53). Collectively, these findings suggest that the ExoS/ChvI system plays an essential role in preparing S. meliloti cells for their transformation from free-living to nitrogen-fixing cells inside the root nodules. The importance of the S. meliloti ExoS/ChvI system was further highlighted by the finding that two of its close homologs are essential for host infections in Brucella abortus and A. tumefaciens (4, 15, 24, 31, 45).

Recent genetic and biochemical data suggest that ExoR, ExoS, and ChvI form a single signal transduction pathway (5, 53). The
ExoR protein has been localized to the periplasm of *S. meliloti* cells (53), as was confirmed by our unpublished data. ExoR has been found to exist in two forms, the 29-kDa full-length precursor form (ExoR<sub>p</sub>) and the 26-kDa mature form without its predicted signal peptide (ExoR<sub>m</sub>), in wild-type *S. meliloti* cells (5). Coimmunoprecipitation of ExoR and ExoS suggested that they form protein complexes (5). Increased expression of the *exoS* gene also led to accumulation of ExoR<sub>p</sub>, suggesting that ExoS stabilizes ExoR in the ExoR-ExoS complex. The ExoR-ExoS interaction was interrupted by single-amino-acid changes in either the ExoR protein or the periplasmic domain of ExoS (5). Taken together, these findings led to a proposed model in which ExoR interacts with ExoS to form a protein complex that keeps ExoS in the off state, resulting in conditions favoring free living, with higher levels of flagellum production and lower levels of succinoglycan production (5).

Expanding on this suggested model, our recent data from genetic analyses suggest that ExoS autoregulates its own expression through the ExoS/ChvI two-component system (33). Loss of functional ExoR protein in the exoR95 mutant leads to upregulation of *exoR* gene expression, along with that of succinoglycan biosynthetic genes (33). This upregulation can be suppressed by single-amino-acid mutations in the ExoS sensing domain, which is consistent with direct ExoR-ExoS interactions. This raises the possibility that ExoR autoregulation through the ExoS/ChvI system is used to modulate the expression of the genes regulated by the ExoS/ChvI system in response to host or environmental signals.

While the current model can explain how ExoR turns off the ExoS/ChvI system, it does not explain how the ExoS/ChvI system is turned on during symbiosis in response to environmental or plant signals. Our new findings, presented here, provide a better understanding of the molecular basis for the ExoR protein’s ability to turn the ExoS/ChvI two-component regulatory system on and off.

### MATERIALS AND METHODS

#### Strains, plasmids, and media

The strains, phages, and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium at 37°C, and *S. meliloti* strains were grown in LB medium supplemented with 2.5 mM MgSO<sub>4</sub> and 2.5 mM CaCl<sub>2</sub>.
(LB-MC) at 30°C (29). LB-MC agar containing 0.02% (wt/vol) calcofluor white M2R (Blue Brighter 28; Sigma) was buffered to pH 7.4 with 10 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid) and used to examine squinoclycan production on agar media (29). The following antibiotics were used at the concentrations indicated: ampicillin, 100 μg ml⁻¹; chloramphenicol, 10 μg ml⁻¹; neomycin, 200 μg ml⁻¹; kanamycin, 25 μg ml⁻¹; spectinomycin, 100 μg ml⁻¹; tetracycline, 10 μg ml⁻¹, and streptomycin, 500 μg ml⁻¹.

**Motility assay.** Bacterial cell motility was examined using swarming plates containing 0.3% agar as described previously, with some modifications (32). Briefly, fresh cell cultures were prepared and diluted to an optical density at 600 nm (OD_600) of 0.1. Then, 2 μl of each diluted culture was inoculated into an LB-MC soft-agar plate and incubated for 2 to 3 days to determine colony size.

**Plant nodulation test.** Alfalfa nodulation assays were carried out on plates as previously described, with slight modifications (29). A set of eight alfalfa seedlings were planted in a square petri dish, and all plants were grown inside the petri dishes. The plants were examined after 4 weeks for the number of nodules to determine overall symbiotic efficiency.

**Expression and purification of S. meliloti ExoR-His for antibody production.** The _S. meliloti_ exoR open reading frame (ORF) was obtained by PCR, using Rm1021 genomic DNA as the template and two PCR primers: exoRfNdeI-1 and exoRxhol-1. The PCR product was digested with NdeI and XhoI and then cloned into PET-16b between NdeI and XhoI restriction sites to generate plasmid pHC615. The His-tagged ExoR protein was purified from BL21(DE3)(plysS, pHC615) with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) induction and using Ni-nitrilotriacetic acid (NTA) purification system under denaturing conditions (Invitrogen). Purification of the His-tagged ExoR protein was confirmed by Coomassie blue-stained SDS-polyacrylamide gels and sent to the Pacific Immunology Company (Ramona, CA) to raise ExoR-specific polyclonal antibodies in rabbits. The specificity of the ExoR-specific polyclonal antibodies was confirmed by Western blotting.

**Immunoblotting.** For immunoblotting, total cellular proteins or purified protein was resolved by SDS-PAGE; transferred to a PVDF (polyvinylidene difluoride) membrane (Bio-Rad); detected with primary antibodies, including ExoR-specific polyclonal antibodies and FLAG-specific monoclonal antibody coupled with alkaline phosphatase (AP) (Novagen); and treated with NBT-BCIP (nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate) (Sigma) to detect AP activities.

**Construction of the exor108-chvi109 double mutant.** The DNA fragment consisting of nucleotides 88 to 433 of the exor ORF was generated by PCR using _S. meliloti_ Rm1021 genomic DNA as the template and two PCR primers: exoRfHindIII-2 and exoRrBsiCI. The PCR product was digested with HindIII and BsrGI and cloned into suicide plasmid pKM19mob2OHM2 (34) to produce pHC508. The resulting plasmid was then conjugated into _S. meliloti_ Rm1021 using helper MT616 in a triparental mating, and a few conjugants were isolated after 6 days. The interrupted _exoR_ gene was transduced from the conjugants into Rm1021, which yielded only one transductant, the _exoR_108 mutant. Insertion of the suicide plasmid into the genome was confirmed by PCR and by sequencing the site of insertion. The _chvl_ gene was amplified from the genome of the _exoR_108 transductant using primers chvlf-27 and chvlr759 and sequenced using primers chvlf293 and chvlr419. The _exoS_ gene was similarly amplified from the genome of the _exoR_108 transductant using primers exoSr61 and exoSr1918 and sequenced using primers exoSr407, exoSr829, exoSr653, exoSr1098, and exoSr1912. One single mutation was found in the _chvl_ gene, but no mutations were found in the _exoS_ gene. The _exoR_108 transductant was thus designated the _exoR_108-chvi109 double mutant. The construct was confirmed by sequencing. The primers used in cloning and sequencing are listed in Table 2, along with all other plasmid constructions described in this paper.

** Constructs expressing ExoR and ExoR without signal peptide.** The DNA fragment containing the _exoR_ promoter and ORF was generated in a PCR using _S. meliloti_ genomic DNA as the template and primers exoR HindIII-1 and exoRxhol-2 and cloned between the HindIII and Xhol sites of a medium-copy-number plasmid, pMB393, to generate pMB510. Similarly, the _exoR_ gene was amplified by PCR using primers _exoR_NdEl-3 and exoRxhol-3 and cloned into pHC93 to generate pHC518, expressing the wild-type ExoR from the lac promoter on the plasmid. The region of the _exoR_ gene without the signal sequence was amplified using primers _exoRNdEl-2 and exoRxhol-3, cloned into plasmid pHC93 to generate pHC641, and used to express ExoRm, the form of ExoR lacking its signal peptide.

**Construction and analysis of ExoR-PhoA fusions.** A set of three _ExoR-PhoA_ fusions were constructed. To construct the fusion of _PhoA_ with the full-length _ExoR_, an XbaI/KpnI DNA fragment containing the _E. coli phoA_ gene (lacking its signal sequence) was prepared by PCR using the genomic DNA of the _S. meliloti_ exoF265: TnphoA mutant as the template and primers phoAXbaI and phoAKpnI. An Xhol/XbaI DNA fragment containing the complete _exoR_ ORF except the stop codon was obtained by PCR using _S. meliloti_ Rm1021 genomic DNA as the template and two PCR primers, exoRixhol and exoRxhol. The two DNA fragments were individually treated with either Xhol/XbaI or XbaI/KpnI and ligated with an Xhol- and KpnI-treated low-copy-number plasmid, pSW213, to generate pHC528. To construct the fusion of _PhoA_ and _ExoR_ without signal peptide, the region of the _ExoR-PhoA_ fusion in pHC528 was amplified without the _ExoR_ signal peptide region using two primers, exoRmXhol and phoAKpnI, and recloned into the same region of pSW213 to create pHC555. To create the fusion of _PhoA_ with _ExoR_ signal peptide, the coding region of the _ExoR_ signal peptide was amplified in a PCR using pHC528 as the template and primers _exoRxhol_ and _exoRsp-phoAr_. The region for _PhoA_ was amplified using primers _exoRsp-phoAf_ and _phoAKpnI_. The two fragments were joined together in an overlapping PCR using primers _exoRxhol_ and _phoAKpnI_. The product of this overlapping PCR was cloned into pSW213 to create pHC533. All three fusions were expressed from an inducible _lac_ promoter on the plasmid with 0.8 mM IPTG.

**Construction and analysis of FLAG-tagged _ExoR_ proteins.** The unique 8-amino-acid FLAG tag (DYKDDDDK; Sigma) was inserted into the C-terminus of the _ExoR_ protein to help track it. To construct the C-terminally tagged _ExoR_, _ExoR-CF_, a DNA fragment containing _ExoR_ and the _flag_ tag, was generated by PCR using _S. meliloti_ genomic DNA as the template and primers _exoR HindIII-1_ and _exoRclagrxhol_, which introduced the _flag_ tag. This DNA fragment was cloned into pMB393 to generate pHC630 and express _ExoR-CF_.

To track FLAG-tagged _ExoR_ proteins, cells of _S. meliloti_ strains expressing the _ExoR-CF_ fusion were collected from exponential-phase cultures; washed with coimmunoprecipitation (CoIP) buffer (36); resuspended in 3 ml CoIP buffer with 1% (wt/vol) Triton X–100, 10 mM MgCl₂, 30 mg lysozyme, and 30 units of DNase I at 4°C for 30 min; and sonicated (550 Sonic Dismembrator; Fisher Scientific) twice on ice (level 2; 4.5 min each time). Cell lysates were centrifuged at 4°C and 13,000 rpm in a microcentrifuge for 5 min. The supernatants were collected, mixed with 20 μl anti-FLAG M2-agarose (Sigma), and incubated overnight at 4°C. The M2-agarose was collected from the reaction mixture by centrifugation at 5,000 rpm and 4°C for 3 min, washed three times with CoIP washing buffer, resuspended in 75 μl of 100 ng μl⁻¹ FLAG peptide, incubated at 4°C for 1 h, and centrifuged at 13,000 rpm for 5 min to collect the supernatant for storage at −20°C. Alternatively, the washed M2-agarose was mixed with 75 μl loading buffer, boiled at 100°C for 5 min, and stored at −20°C

**Direct isolation of FLAG-tagged _ExoR_CF_ from _S. meliloti_ periplasm.** An overnight culture of the _S. meliloti_ exoR95 (pHC630) strain was diluted 1:100 in 1 liter of fresh LB-MC–spectinomycin and further incubated at 30°C with shaking to an OD_600_ of 0.3. Cells were collected, washed twice in Tris-buffered saline (TBS) (20 mM Tris, 0.2 mM NaCl, pH 7.5), resuspended in TBS with 0.5 M sorbitol for 10 min at room temperature, collected by centrifugation, resuspended in sterile water for 10 min on ice, and removed by two consecutive centrifugations (10 min
The DNA sequence encoding FLAG peptide is in boldface.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligonucleotide nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>exoRNdel-1</td>
<td>5'-GGATTCATATGAGGGCAGGTGGATGGAAGTC-3'</td>
</tr>
<tr>
<td>exoRxhol-1</td>
<td>5'-CCGGCTAGATCTGCGGAGCCTCGG-3'</td>
</tr>
<tr>
<td>exorcflagrXhoI</td>
<td>5'-CCGGCTAGATCTGCGGAGCCTCGG-3'</td>
</tr>
<tr>
<td>exorRfNdeI-2</td>
<td>5'-GGGGAATTGCCATATGACGACGACG-3'</td>
</tr>
<tr>
<td>exorRsp-phoAr</td>
<td>5'-CCGGCTAGATCTGCGGAGCCTCGG-3'</td>
</tr>
<tr>
<td>exorRL81Ar</td>
<td>5'-CCGGCTAGATCTGCGGAGCCTCGG-3'</td>
</tr>
<tr>
<td>exorRW79Ar</td>
<td>5'-CCGGCTAGATCTGCGGAGCCTCGG-3'</td>
</tr>
<tr>
<td>exorRW79Af</td>
<td>5'-CCGGCTAGATCTGCGGAGCCTCGG-3'</td>
</tr>
<tr>
<td>exorRW79rAf</td>
<td>5'-CCGGCTAGATCTGCGGAGCCTCGG-3'</td>
</tr>
<tr>
<td>exorR90w241f</td>
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</tr>
<tr>
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</tr>
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<td>chvr759</td>
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</tr>
<tr>
<td>chvif239</td>
<td>5'-CCGGCTAGATCTGCGGAGCCTCGG-3'</td>
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<tr>
<td>chvr419</td>
<td>5'-CCGGCTAGATCTGCGGAGCCTCGG-3'</td>
</tr>
<tr>
<td>exosf-61</td>
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</tr>
<tr>
<td>exosf1918</td>
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</tr>
<tr>
<td>exosf1912</td>
<td>5'-CCGGCTAGATCTGCGGAGCCTCGG-3'</td>
</tr>
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The MS-MS data generated from nano-LC/electrospray ionization (ESI)-based IDA analysis were submitted to Mascot 2.2 for database searching using an in-house-licensed Mascot local server, and a search was performed to query the Swiss-Prot database (taxonomy, Proteobacteria) with one missed cleavage site by trypsin allowed. The peptide tolerance was set to 1.5 Da, and the MS-MS tolerance was set to 0.6 Da. Carbamidomethyl modification of cysteine and oxidation of a methionine were set as variable modifications. Only significant scores for the peptides
ExoR,268 aa

![Diagram of ExoR protein showing signal peptide (SP), four putative Sel1 protein-protein interaction domains (SEL1), a FLAG-tagged ExoR protein (ExoR-CF), two mutated ExoR proteins (ExoR95 and ExoR108), the proteolysis region of ExoR, the amino acids that are conserved (in black-shaded letters) (Fig. 6) around the proteolysis region, and the positions of the 3 amino acids that were mutated to alanine (A) for functional analyses. The amino acid sequences of the C-terminal FLAG tag (CF), the ExoR95 C terminus, and the target region (81 to 87) of ExoR proteolysis are shown.](http://www.matrixscience.com/help/scoring_help.html#PBM)

**FIG 1** Schematic representation of the ExoR protein showing the signal peptide (SP), four putative Sel1 protein-protein interaction domains (SEL1), a FLAG-tagged ExoR protein (ExoR-CF), two mutated ExoR proteins (ExoR95 and ExoR108), the proteolysis region of ExoR, the amino acids that are conserved (in black-shaded letters) (Fig. 6) around the proteolysis region, and the positions of the 3 amino acids that were mutated to alanine (A) for functional analyses. The amino acid sequences of the C-terminal FLAG tag (CF), the ExoR95 C terminus, and the target region (81 to 87) of ExoR proteolysis are shown.

defined by Mascot probability analysis (http://www.matrixscience.com/help/scoring_help.html#PBM) greater than "identity" were considered for the peptide identification.

**Construction of an ExoR<sub>95</sub>-expressing plasmid.** To construct a translational fusion of the exoR signal peptide and the ExoR C-terminal proteolytic product (ExoR<sub>95</sub>), the exoR signal peptide coding region, along with the exoR promoter region and the ExoR<sub>108</sub> (amino acids 81 to 268) coding region, was obtained individually from two PCRs. The primers used to amplify the coding region for the exoR promoter and the ExoR signal peptide were exoRHindIII-1 and exoR90w241r, and those used to amplify the coding region for ExoR<sub>95</sub> were exoR90w241f and exoRXhoI-2. After PCR and purification, the two fragments were ligated together via recombinant PCR using primers exoRHIhindIII-1 and exoRXhoI-2. This PCR product was then purified and digested with restriction enzymes HindIII and XhoI and ligated with the vector pMB393 digested with the same enzymes to generate plasmid pH567.

**Construction of plasmids expressing ExoR protein with single-amino-acid mutations.** Single-amino-acid mutations of ExoR were introduced via PCR site-directed mutagenesis. To construct pH571 with the exoR L81A mutation, two separate PCRs were run using primer pairs exoRHindIII-1/exoRL81Ar and exoRL81Af/exoRXhol-2 and plasmid pH510 DNA as the template. Primers exoRL81Ar and exoRL81Af introduced a codon change resulting in an L-to-A mutation at position 81 of the ExoR protein. The two PCR DNA fragments were ligated together via recombinant PCR using primers exoRHIhindIII-1 and exoRXhoI-2. This PCR product was then purified and digested with restriction enzymes HindIII and Xhol and ligated with the vector pMB393 digested with the same enzymes to generate plasmid pH567.

Using a similar approach, plasmid pH572 was constructed with the W79A mutation created by primers exoRW79Ar and exoRW79Af, and pH573 was constructed with the Y87A mutation created by primers exoRY87Ar and exoRY87Af.

**RESULTS**

**Extra protein bands in ExoR profiles.** The ExoR protein auto-regulates its own expression through the ExoS/ChvI system (33), and it has been shown to exist in a 29-kDa precursor form (ExoRp), a 25-kDa active mature form (ExoRm), and the target region (81 to 87) of ExoR proteolysis are defined by Mascot probability analysis (http://www.matrixscience.com/help/scoring_help.html#PBM) greater than "identity" were considered for the peptide identification.

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The exoR95 mutant is a transposon Tn<sub>5</sub> insertion mutant; the site of the Tn<sub>5</sub> insertion has been genetically mapped to the end of the exoR gene (41). To determine the size and amino acid sequence of the ExoR95 protein, the site of the transposon insertion was determined. Tn<sub>5</sub> was found to be inserted between codons 248 and 249, replacing the original 20 C-terminal amino acids of ExoR (DDRRVAITMSQNMHLQDDD) with a new set of 9 amino acids (ADSYTQVAS) in the ExoR95 protein. This suggested that if the ExoR95 protein is synthesized and exported to the periplasm like the wild-type ExoR, it should exist in 28-kDa protein. Western blot analysis showed that the exoR95 mutant not only has the predicted 28-kDa ExoR95<sub>p</sub> and 25-kDa ExoR95<sub>m</sub> forms (Fig. 2), but also the 21- and 24-kDa proteins that were found in the wild-type Rm1021 cells. It is also interesting that the 20-kDa protein is missing from the exoR95 mutant. The 21- and 24-kDa proteins are smaller than the 25-kDa ExoR95<sub>m</sub>, which makes it possible in theory for them to be derivatives of the 25-kDa ExoR95<sub>m</sub> protein. Therefore, a strain with a smaller, or no, ExoR protein was needed to help identify the origins of the 20-, 21-, and 24-kDa proteins.

**Construction of an exoR mutant for biochemical analysis.** After multiple attempts, we were able to obtain only one exoR mutant—the exoR108 mutant, resulting from the insertion of plasmid phC508 carrying part of the exoR ORF. The ExoR108 protein is predicted to be a fusion of 144 N-terminal amino acids from ExoR and 51 C-terminal amino acids from the inserted suicide plasmid (Fig. 1). The exoR108 mutant overproduced succi-
noglycan and formed nonswimming colonies, similar to the exoR95 mutant. These exoR108 mutant phenotypes were complemented by the plasmid pHC510 expressing wild-type ExoR protein, suggesting that the exoR108 mutation is a loss-of-function mutation (Fig. 3A and B).

The difficulties in obtaining an exoR mutation, and prior findings of exoR suppressor mutations in the exoS and chvI genes, prompted us to check exoS and chvI in the exoR108 mutant. Interestingly, while there was no mutation in the exoS gene, there was a single G-to-A nucleotide change at position 325 in the chvI gene in the exoR108 mutant. This mutation results in a change in amino acid 109 of the ChvI protein from V to M, so the exoR108 mutant is in fact an exoR108-chvI109 double mutant. It was possible to transduce the exoR108 mutation from the exoR108-chvI109 mutant into wild-type Rm1021 expressing the wild-type exoR gene from plasmid pH510, but not into Rm1021 itself. This raised the possibility that the exoR gene can only be interrupted to generate the exoR108 mutation in the presence of either a plasmid-borne copy of the exoR gene or the chvI109 mutation. These findings suggest that the possibility that the chvI109 mutation could contribute to the phenotype of the exoR108-chvI109 double mutant cannot be ruled out, and the mutant could therefore only be used for biochemical analyses of ExoR proteins in this study.

A novel 20-kDa form of the ExoR protein. Three proteins at 29, 26, and 20 kDa stood out clearly in the ExoR protein profiles of the exoR108-chvI109 double mutant with plasmid pHC510 expressing wild-type ExoR protein, while the intensities of the 21- and 24-kDa bands remained the same as for the exoR108-chvI109 double mutant without plasmid pH510 (Fig. 2). The positions of the 29-, 26-, and 20-kDa proteins matched those of the three protein bands in wild-type strain Rm1021 (Fig. 2). Since the 21- and 24-kDa proteins were present in wild-type Rm1021, as well as in the exoR95 and exoR108 mutants, they are most likely nonspecific proteins. Together, these findings suggest that the ExoR protein exists in three different forms, 29, 26, and 20 kDa. The finding of the previously unknown 20-kDa ExoR protein suggests that ExoR could be the target of proteolysis.

ExoRm is digested in the periplasm. The 20-kDa ExoR protein, designated ExoRm, could be the proteolysis product of either ExoRp in the cytoplasm or ExoRm in the periplasm. To find a way to restrict ExoR in either the cytoplasm or periplasm, the role of its signal peptide was further examined. The entire ExoR protein (ExoRp), ExoR without the signal peptide (ExoRm), and the ExoR signal peptide were separately fused to PhoA (Fig. 4A).
presence of the signal peptide was sufficient for the ExoR-PhoA fusion and PhoA to be exported to the periplasm, where it displayed phosphatase activity, as indicated by the blue color of the colonies on medium containing XP (5-bromo-4-chloro-3-indolylphosphate) (Fig. 4B). In the absence of signal peptide, the ExoRm-PhoA fusion protein remained inside the cells, as indicated by the white color of the colonies (Fig. 4B).

To help determine the site of ExoRm proteolysis, wild-type ExoR and ExoRm were expressed in the exoR95 mutant from plasmids pHC518 and pHC641 to deliver ExoRm to the periplasm or cytoplasm, respectively. When wild-type ExoR was expressed in the exoR95 mutant from plasmid pH518, all three forms of the wild-type ExoR—ExoRm, ExoRm, and ExoRm—were clearly visible, in addition to ExoR95m and ExoR95m (Fig. 4C). In contrast, when ExoRm was expressed in the exoR95 mutant from plasmid pHC641, the ExoRm protein, as well as ExoR95m and ExoR95m, was found, but very little ExoRm protein was detected (Fig. 4). This suggests that ExoRm is very ineffectively processed inside the cytoplasm. Taken together, these findings suggest that ExoRm is digested efficiently in the periplasm to yield the 20-kDa form. This conclusion was further confirmed by direct isolation and sequencing of the ExoR proteolysis product from the periplasm, as described below.

Functional analyses of C-terminally FLAG-tagged ExoR protein. To determine whether the 20-kDa form of ExoR shares the same C terminus with ExoRm, ExoR-CF, an ExoR protein with an 8-amino-acid C-terminal FLAG tag (DYKDDDDK), was generated by fusing the FLAG tag to the ExoR C terminus (Fig. 1). ExoR-CF was expressed from plasmid pH630 with the native exoR promoter. Our results showed that ExoR-CF was as effective as the wild-type ExoR in restoring the swimming activity (Fig. 1). ExoR-CF was expressed from plasmid pH630 with the native exoR promoter. Our results showed that ExoR-CF was as effective as the wild-type ExoR in restoring the swimming activity (Fig. 1B) and nodulation of alfalfa plants of the exoR95 mutant, as determined by the percentage of pink nodules (Fig. 3C). These findings suggest that the biochemical analysis of ExoR-CF should reflect the properties of the wild-type ExoR protein.

The C terminus of the 20-kDa form of ExoR. The C terminus of the 20-kDa form of ExoR could be determined by identifying which form of ExoR protein retains the FLAG tag. Total proteins from the exoR95 mutant expressing ExoR and ExoR-CF proteins from plasmids pH510 and pH630, respectively, were prepared, immunoprecipitated with FLAG-specific monoclonal antibody, and probed with either our ExoR polyclonal antibodies (Fig. 5A) or the FLAG-specific monoclonal antibody (Fig. 5B). Wild-type ExoR protein without a FLAG tag could not be precipitated with the FLAG-specific antibody, so it was not detected by either antibody. Our analysis of C-terminally FLAG-tagged ExoR-CF showed that all three different forms of ExoR were detected by both ExoR and FLAG antibodies (Fig. 5). This suggests that ExoRm, ExoRm, and ExoRm were all carrying the FLAG tag. Altogether, these findings suggest that the 20-kDa form of ExoR derives from the C-terminal side of the ExoR protein (hence its designation, ExoR20).

The N terminus of the 20-kDa ExoR fragment and the site of ExoR proteolysis. To identify the N-terminal amino acid of ExoR20, which is also the site of ExoR proteolysis, the C-terminally FLAG-tagged ExoR-CF proteins were isolated directly from the periplasm of S. meliloti cells using osmotic shock and immunoprecipitation with commercial monoclonal FLAG tag antibody. ExoR20, ExoR20, with a C-terminal FLAG tag, was first isolated directly from a crude preparation of periplasmic proteins by immunoprecipitation and then resolved by SDS-PAGE, transferred to a PVDF membrane, and stained with Coomassie blue. A piece of membrane containing ExoR20-CF was used to identify its N-terminal amino acid by peptide mapping.

The results of the peptide mapping showed that the N-terminal amino acid of ExoR20-CF is L and that it is amino acid 81 in the ExoR protein. The confidence in this finding was measured by Mascot probability analysis (http://www.matrixscience.com/help/scoring_help.html#PBM). A Mascot value of 59 represents 99% confidence. The Mascot value for L as the N-terminal amino acid was 94, indicating more than 99% confidence in the identification. Interestingly, amino acids 84, 85, 86, and 87 were also identified as N-terminal amino acids, with Mascot values between 85 and 111. This suggested that ExoR proteolysis could take place between amino acids 80 and 87 of the ExoR protein to yield a mixture of 181- to 188-amino-acid-long peptides (Fig. 1). These peptides could be the result of a single digestion between amino acids 80 and 81 followed by additional digestion, or they could be the result of a single random digestion within the region of amino acids 80 to 87. The preparation method for peptide mapping used here precluded us from determining which fragment was the dominant form. Taken together, these findings suggest the strong likelihood of ExoRm protein being further digested between amino acids 80 and 87 to yield ExoR20, a set of 181- to 188-amino-acid (19.9- to 20.6-kDa) peptides.

A large number of ExoR orthologs have been discovered through genome sequencing, although few of them have been characterized in detail. Sixteen ExoR orthologs were aligned based on their overall amino acid identity, ranging from 97% to 51% (Fig. 6). Despite the dramatic decrease in the overall amino acid identity, however, the amino acid sequence around the ExoR proteolysis sites remains highly conserved among the ExoR orthologs (Fig. 6). This raises the possibility that ExoR proteolysis is conserved among other ExoR orthologs and that proteolysis could be a common molecular signaling mechanism for ExoR proteins in different bacteria.

ExoR20 does not function in the ExoS/ChvI system. The discovery that both ExoRm and ExoRm are stably maintained in the wild-type Rm1021 cells, and that ExoRm is absent in the loss-of-function exoR95 mutant, raised the possibility that ExoRm is the functional form of the protein. To examine the function of
ExoR$_{c20}$ directly, the longest form of ExoR$_{c20}$ with 188 amino acids, was fused to the 30-amino-acid ExoR signal peptide to generate SP-ExoR$_{c20}$ expressed from pHC567. When total proteins from the exoR108-chvI109 mutant expressing SP-ExoR$_{c20}$ were probed with ExoR polyclonal antibodies, both SP-ExoR$_{c20}$ and ExoR$_{c20}$ were detected (Fig. 7A). This suggests that SP-ExoR$_{c20}$ is expressed, processed, and stably maintained without its signal peptide in the periplasm.

To test the function of ExoR$_{c20}$, the SP-ExoR$_{c20}$ protein was expressed in the wild-type strain Rm1021 and the exoR95 mutant. The presence of the SP-ExoR$_{c20}$-expressing plasmid pHC567 did not change either the succinoglycan or the swimming phenotype for either wild-type Rm1021 or the exoR95 mutant (Fig. 7C and D). These results, along with the finding that the wild-type ExoR protein was able to complement the phenotypes of the exoR95 mutant (Fig. 2), suggest that the ExoR proteolysis product, ExoR$_{c20}$, does not function in regulating the production of succinoglycan or flagella.

**ExoR proteolysis and regulatory functions are altered by point mutations.** Our finding of functional ExoR$_{m}$ and nonfunctional ExoR$_{c20}$ coexisting in the periplasm raised the possibility that the amount of ExoR$_{m}$ is maintained at a certain level and that the amino acids and the site of proteolysis are marked based on the identities between ExoR orthologs. Conserved amino acids are shaded. The overall amino acid identities between $S$. meliloti ExoR orthologs are listed. The positions of the amino acids and the site of proteolysis are marked based on the $S$. meliloti ExoR protein. The following species are shown: $S$. meliloti Rm1021 (S. m.), Sinorhizobium medicae WSM419 (S. m. w.), Rhizobium sp. strain NGR234 (R. sp.), Agrobacterium vitis 54 (A. v.), Agrobacterium tumefaciens strain C58 (A. t.), Agrobacterium radiobacter K84 (A. r.), Rhizobium leguminosarum bv. trifolioli WSM2304 (R. l.), Heflea photrotropaic DFL-43 (H. p.), Rhizobium etli CIAT652 (R. e.), Mesorhizobium sp. strain BNC1 (M. sp.), Ochrobactrum intermedium LM3 3301 (O. i.), Ochrobactrum anthropi ATCC 49188 (O. a.), Brucella abortus strain 2308 A (B. a.), Brucella ceti strain Cudo (B. c.), Brucella ovis ATCC 25840 (B. o.), and Brucella melitensis 16 M (B. m.).

![FIG 6](https://example.com/figure6.png) **FIG 6** Amino acid sequence alignment of ExoR proteolysis regions from 16 ExoR orthologs. Conserved amino acids are shaded. The overall amino acid identities between $S$. meliloti ExoR and its orthologs are listed. The positions of the amino acids and the site of proteolysis are marked based on the $S$. meliloti ExoR protein. The following species are shown: $S$. meliloti Rm1021 (S. m.), Sinorhizobium medicae WSM419 (S. m. w.), Rhizobium sp. strain NGR234 (R. sp.), Agrobacterium vitis 54 (A. v.), Agrobacterium tumefaciens strain C58 (A. t.), Agrobacterium radiobacter K84 (A. r.), Rhizobium leguminosarum bv. trifolioli WSM2304 (R. l.), Heflea photrotropaic DFL-43 (H. p.), Rhizobium etli CIAT652 (R. e.), Mesorhizobium sp. strain BNC1 (M. sp.), Ochrobactrum intermedium LM3 3301 (O. i.), Ochrobactrum anthropi ATCC 49188 (O. a.), Brucella abortus strain 2308 A (B. a.), Brucella ceti strain Cudo (B. c.), Brucella ovis ATCC 25840 (B. o.), and Brucella melitensis 16 M (B. m.).

![FIG 7](https://example.com/figure7.png) **FIG 7** (A) Biochemical and functional analysis of the ExoR proteolysis product ExoR$_{c20}$, which was expressed from pHC510 (pR) and pHC567 (pSP-R$_{c20}$) in the exoR108-chvI109 mutant for ExoR and SP-ExoR and probed in a Western blot. (B to D) The effects of the presence of ExoR$_{c20}$ on succinoglycan production was measured by calcofluor fluorescence (B and C), and its effects on swimming ability were measured by the sizes of the colonies (D).

ExoR$_{c20}$, the longest form of ExoR$_{c20}$ with 188 amino acids, was fused to the 30-amino-acid ExoR signal peptide to generate SP-ExoR$_{c20}$ expressed from pHC567. When total proteins from the exoR108-chvI109 mutant expressing SP-ExoR$_{c20}$ were probed with ExoR polyclonal antibodies, both SP-ExoR$_{c20}$ and ExoR$_{c20}$ were detected (Fig. 7A). This suggests that SP-ExoR$_{c20}$ is expressed, processed, and stably maintained without its signal peptide in the periplasm.

To test the function of ExoR$_{c20}$, the SP-ExoR$_{c20}$ protein was expressed in the wild-type strain Rm1021 and the exoR95 mutant. The presence of the SP-ExoR$_{c20}$-expressing plasmid pHC567 did not change either the succinoglycan or the swimming phenotype for either wild-type Rm1021 or the exoR95 mutant (Fig. 7C and D). These results, along with the finding that the wild-type ExoR protein was able to complement the phenotypes of the exoR95 mutant (Fig. 2), suggest that the ExoR proteolysis product, ExoR$_{c20}$, does not function in regulating the production of succinoglycan or flagella.

**ExoR proteolysis and regulatory functions are altered by point mutations.** Our finding of functional ExoR$_{m}$ and nonfunctional ExoR$_{c20}$ coexisting in the periplasm raised the possibility that the amount of ExoR$_{m}$ is maintained at a certain level and that any changes to the level of ExoR$_{m}$ alter the function of the ExoR protein. To test this possibility, two highly conserved amino acids at positions 79 and 81 and one nonconserved amino acid at position 87 were changed individually to A (alanine) (Fig. 1). When these three mutant ExoR proteins, ExoRW79A, ExoRL81A, and ExoRY87A, were expressed from plasmids pHC571, pHC572, and pHC573, respectively, in the exoR108-chvI109 double mutant, the level of the ExoR$_{m}$ form was significantly reduced for the ExoRL81A mutant and slightly reduced for the ExoRW79A mutant but not changed for the ExoRY87A mutant (Fig. 8A). This suggests that the L81A mutation may have reduced the amount of the ExoR$_{m}$ form of ExoR mutant protein in the cells.

The regulatory functions of the three mutated ExoR proteins were examined by assessing their abilities to complement the succinoglycan-producing (represented by calcofluor brightness) and nonswimming phenotypes of the exoR95 mutant (Fig. 8C and D). Compared to the wild-type ExoR protein, the ExoRL81A protein was not able to complement either the succinoglycan-producing or nonswimming phenotype of the exoR95 mutant, and it is therefore a loss-of-function mutation. Both the ExoRW79A and ExoRY87A proteins partially complemented both the succinoglycan-producing and nonswimming phenotypes of the exoR95 mutant. ExoRY87A was more effective than ExoRW79A in complementing the succinoglycan-producing phenotype. These results suggest that both ExoRW79A and ExoRY87A are functional and that ExoRY87A is more effective than ExoRW79A but less effective than wild-type ExoR.

The combined biochemical and functional analyses of the three mutant ExoR proteins suggest that the level of ExoR$_{m}$ is linked to the regulatory function of the ExoR protein. This raises the possibility that ExoR proteolysis can be modulated by environmental or plant signals to regulate the production of succinoglycan, flagella, and many other cellular products required for symbiosis.

**DISCUSSION**

Recent publications have shown that the $S$. meliloti ExoR protein most likely functions as the repressor of the ExoS sensor, as part of the ExoR autoregulation pathway, which allows it to indirectly regulate the expression of a large number of genes required for host invasion and symbiosis (5, 33, 33). Both genetic and biochemical data appear to support the model that ExoR interacts with the ExoS periplasmic sensing domain directly to suppress ExoS, keeping it in an off state. It follows that ExoR suppression of ExoS would have to be relieved during nodulation so that the expression of the invasion and symbiosis genes could be turned on.
to support the noduleation. However, the molecular mechanism mediating the relief of ExoR suppression of ExoS is not clear.

One simple and attractive model for the relief of ExoR suppression is a reduction in the amount of ExoR protein in its mature and active form, ExoRm, by changing it to an inactive form through proteolysis or modification. To monitor such changes in ExoRm, on a Western blot, antibodies that can recognize the entire ExoR protein and an S. meliloti strain to provide the genetic background with no ExoR protein are required. While generating ExoR-specific polyclonal antibodies was relatively straightforward, engineering an S. meliloti strain with no ExoR proved to be challenging.

Two complementary approaches were followed to find a clean background for analysis of the ExoR protein. The first was to determine the status of ExoR in the original loss-of-function exoR95 mutant. Our analysis showed that the mutated ExoR95 protein can be easily found in two forms, ExoR95w and ExoR95m. This makes it more difficult to monitor the changes in wild-type ExoR protein, since these two forms are just slightly smaller than the wild type, as predicted based on the site of the exoR95 mutation. The second approach, which was to generate an ExoR deletion mutation, turned out to be much more difficult. The only new exoR mutation generated from this effort was the exoR108-chvI109 double mutant with a plasmid insertion in the exoR108-chvI109 gene. The exoR108 mutation can be transduced from the exoR108-chvI109 double mutant into wild-type strain Rm1021, but not into Rm1021 exoR95 mutant. The presence of ExoRc20 in both strains was confirmed by Western blotting. The presence of stably maintained ExoRc20 in wild-type Rm1021, but not in the loss-of-function exoR95 mutant, raised the question of whether ExoRc20 functions in the ExoR-ExoS/ChvI signal transduction pathway. To address this question, the longest form of ExoRc20 was fused directly to the ExoR signal peptide and expressed in the wild-type Rm1021 and the exoR95 mutant. The presence of ExoRc20 in both strains was confirmed by Western blotting. The presence of ExoRc20 did not alter the succinoglycan and swimming phenotypes of either the wild type or the exoR95 mutant. This suggests that ExoRc20 does not play any significant role in regulating succinoglycan or flagellum production.

More importantly, it also suggests that digesting ExoRm to ExoRc20 might function as a molecular mechanism regulating the amount of ExoRc20 in the periplasm.

For proteolysis of ExoRc20 to serve as a mechanism regulating the function of ExoR, modulation of the amount of ExoRc20 should change the regulatory function of ExoR. To test this hypothesis, the highly conserved amino acid leucine (L) at the site of proteolysis was changed to alanine (A), generating ExoRL81A. In addition, similar site-directed mutations were generated for another conserved amino acid, giving ExoRW79A, and a nonconserved
Our model predicts that plant or environmental signals would alter the rate of ExoRm proteolysis, thereby changing the level of ExoS suppression of ExoS. This would allow S. meliloti cells to modulate the production of succinoglycan and flagella, as well as the expression of many other ExoR-ExoS/ChvI pathway-regulated genes, to support the establishment of symbiosis in response to the presence of plant host or environmental signals. Based on this prediction, we are currently screening for conditions that can change the level of succinoglycan production by the wild-type Rm1021.

Close homologs of ExoS, as well as of ExoS and ChvI, of the ExoR-ExoS/ChvI signal transduction pathway have been found in the genomes of more than 40 different bacterial species. The regulatory mechanisms and the genes regulated by these systems are, with a few exceptions, unknown. When ExoR homologs are aligned to the S. meliloti ExoR protein in order of decreasing overall homology from 93% to 50%, the levels of homology around the region of proteolysis remain unchanged. The high levels of conservation of the protein sequence around the ExoRm proteolysis region raise the possibility that ExoR proteolysis is a common molecular mechanism mediating bacterial sensing in the presence of their hosts or of changes in their environment.

Bacterial periplasmic proteases have been shown to participate in bacterial sensing of environmental signals in several systems. An E. coli membrane-bound protease, DegS, which is activated by unassembled outer membrane porins, cleaves the periplasmic domain of the membrane-anchored regulator RseA (1, 49). This triggers further cleavage of RseA by a metalloprotease, YaeL, to release $\sigma^+$, which normally attaches to RseA (49). The $\sigma^+$ turns on the expression of stress-related genes (49). In the case of a polarity determinant of Caulobacter, PodJ, the periplasmic domain of PodJ is also regulated through its cleavage into small fragments by a periplasmic protease, PerP (6). The example that is closest to S. meliloti ExoR is the function and regulation of the E. coli periplasmic adaptor protein, CpxP, which is involved in sensing pH variations to regulate membrane lipid composition (9, 26, 40, 54). The CpxP protein interacts with and inhibits the periplasmic sensing domain of the CpxA protein, the sensor of the CpxA/CpxR two-component system (14, 46, 54, 56). The periplasmic serine protease DegP is activated by general envelope disruptions, including pH changes, and cleaves the CpxP protein, thereby removing CpxP from the CpxA sensor (26). This results in the activation of the CpxA sensor and the expression of its regulated genes (26, 54). Our finding that ExoR is the subject of proteolysis in the periplasm suggests a regulatory role similar to those of RseA, PodJ, and CpxP.

While it has been well documented that ExoR, ExoS, and ChvI
play essential roles in symbiosis, it is not known what environmental signals are transmitted through the ExoR-ExoS/ChvI signal transduction pathway. The factors that might function upstream of ExoR are also unknown. Our finding of ExoR proteolysis in the periplasm suggests a protease(s) as a key factor upstream of ExoR. The proteolysis of ExoR might be regulated by other proteins in the periplasm through protein-protein interactions or by other enzymes that modify ExoR. These possibilities will be further investigated to gain more insight into the regulation of ExoR and the function of the ExoR-ExoS/ChvI signal transduction pathway. The results of our analysis of ExoR will no doubt be helpful in understanding and combating the pathogenicities of A. tumefaciens and B. abortus, as well as many other host-interacting bacteria that rely on homologs of the ExoR-ExoS/ChvI signal transduction pathway.

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